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(71) Applicant (*for all designated States except US*): MEDICAGO INC. [CA/CA]; 2480, rue Hochelaga, Sainte-Foy, Québec G1K 7P4 (CA).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): VÉZINA, Louis-Philippe [CA/CA]; 206, Route 138, Neuville, Québec G0A 2R0 (CA). D'AOUST, Marc-André [CA/CA]; 939, avenue Manrèse, App. 2, Québec, Québec G1S 2W9 (CA).

(74) Agents: CÔTÉ, France et al.; Swabey Ogilvy Renault, Suite 1600, 1981 McGill College Avenue, Montréal, Québec H3A 2Y3 (CA).

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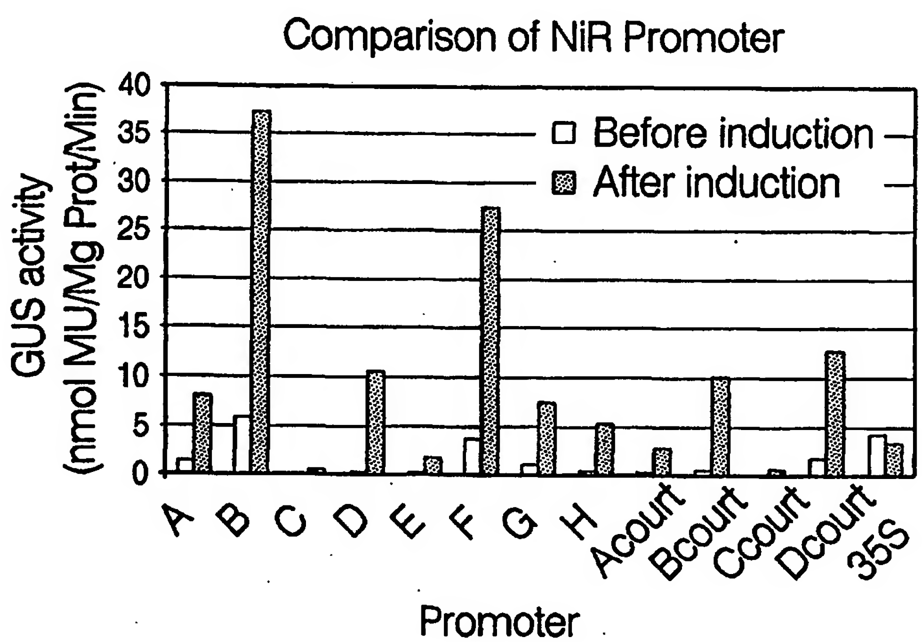
(54) Title: METHOD FOR REGULATING TRANSCRIPTION OF FOREIGN GENES



(57) Abstract: The present invention relates to a method of regulating the transcription of transgene in genetically-modified organisms. More specifically, the invention relates to the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of nitrogen.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD FOR REGULATING TRANSCRIPTION OF FOREIGN GENES

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a method of regulating the transcription of transgene in genetically-modified organisms. More specifically, the invention relates to the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of nitrogen. Preferably, these constructs are used in transgenic leguminous plants (for example soybean, alfalfa, clover, birdsfoot trefoil, beans, peas, peanuts) where growth is not impaired by lack of mineral nitrogen, and in which induction of expression could be performed at any given time during development, through the addition of a suitable nitrogen source. In a broader perspective, the invention could be used to induce expression of any given transgene through the addition of any nitrogen source, provided that the organism can be grown adequately in the absence of this nitrogen inducer; as an example within the plant kingdom, duckweed (Lemna minor) can adapt to grow either on nitrate or ammonium as nitrogen source; transgenic duckweed could therefore be grown on nitrate as a sole nitrogen source and expression of the transgene triggered by the addition of ammonium, provided that the cassette contains a promoter from a native gene for which expression is turned on by the addition of ammonium. The invention therefore provides a means of regulating the expression of a transgenic trait in any organism through the addition of various nitrogenous inducer.

(b) Description of Prior Art

Nitrogen is a molecule essential to life. All living organism need nitrogen in order to synthesize amino acids, the building blocks of proteins, and nucleotides, the building blocks of nucleic acids. It is Ammonium nitrate is the preferred form of mineral nitrogen provided to crops in the form of fertilizer. Nitrate-nitrogen is first reduced to nitrite and then to ammonium through the activity of a metabolic pathway common to most herbaceous plants. Depending on the species, part or all of the absorbed nitrate will move to leaf cells through the xylem before it is

reduced to ammonium. Ammonium, or other reduced forms of nitrogen are also absorbed (although usually at lower rates) by the root system but their assimilation does not require reduction. These newly absorbed ammonium or ammonium-containing molecules join the endogenous pools in the cells which is formed by ammonium cycling through amino acids and other nitrogenous molecules. Some species do not metabolize nitrate-nitrogen easily and therefore cannot rely on nitrate as sole nitrogen source; many coniferous species fall into this latter category. Legumes and other symbiotic plant species form a third large class of nitrogen user within the plant kingdom; they form a metabolic alliance with a microbial organism through which they can fix gaseous nitrogen. This reduced nitrogen is used efficiently by the plant for growth, and therefore, these crops can develop independently of the availability of mineral nitrogen in the soil.

Many microbes and wild plant species will adapt extensively to availability of nitrogen sources and can therefore complete their life cycle in the absence of one molecular form of nitrogen, which they could use exclusively and efficiently if available in another growing environment. As for most assimilatory pathways, nitrogen assimilation is tightly regulated in cells. As an example, the expression of genes encoding nitrate reductase (NaR) and nitrite reductase (NiR), which are responsible for the reduction of nitrate to ammonium, has been extensively described in various microbial and plant species (for a review, see Miflin and Lea, Books 5 and 12, in *The Biochemistry of plants*). Although nitrate is not the only regulatory molecule involved in the control of NaR and NiR expression, its presence is essential to initiate the cascade of transduction that eventually leads to sustained transcription and translation of these genes. It has been shown that expression of NaR and NiR genes is repressed in leguminous plants when they are grown in the absence of mineral nitrogen

NiR promoters have been characterized in some plant species (Back et al., 1991, *Plant Molecular Biology* 17:9-18; Sander et al., 1995, *Plant Molecular Biology* 27:165-177). Inducibility of these promoters have also been characterized using marker genes in transgenic plants, where it

was shown that availability of nitrate is required for full activation of transcription.

Assimilatory pathways for other nitrogen sources have also been described, and promoters for genes involved in some of these pathways have also been characterized.

Genetic transformation of microbes have been used for more than 15 years to produce useful recombinant molecules, and applications in the pharmaceutical, cosmaceutical and dermaceutical industries are being currently exploited. This technology has expanded from microbes to plants and animals in the last ten years with the development of techniques required to adapt this general concept to complex eukaryotic organisms. Basically a gene encoding for a protein of interest or a gene encoding for an enzyme responsible for a modification of a metabolic pathway that leads to a molecule of interest, is linked in an appropriate fashion to cis-and trans-acting regulatory sequences, and transferred to a target cell where it is incorporated in the molecular machinery (in a transitory or stable fashion). The transgenic cell, or a tissue or organism regenerated from the transgenic cell will then perform transcription and translation of the transgene and therefore be enabled to accumulate the protein of interest or to perform the new metabolic reaction through the activity of the enzyme of interest.

The emerging industry of molecular farming (production of recombinant molecules in animals or crops) is one of the most promising industry of the coming century. Its promise is to provide safe and renewable molecule factories for the industry. Among the applications that are currently developed are the production of low-cost monoclonal antibodies for therapeutic and diagnostic uses, the production of unlimited amounts of hormones, cytokines and other bio-active molecules for the treatment of chronicle or lethal diseases, the production of bio-safe substitutes for various blood components, the production of unlimited amounts of processing enzymes for the food and pulp industry, the production of low-cost enzymes for waste treatments, and the production of safe bio-active molecules for the cosmetic industry.

Limitations to the application of this technology has often come from the inability of transgenic organisms to accumulate adequate

amounts of the recombinant product, as a result of low transcription rates, improper splicing of the messenger, instability of the foreign mRNA, poor translation rates, hyper-susceptibility of the recombinant protein to the action of endogenous proteases or hyper-susceptibility of the recombinant organism to the foreign protein which result in improper and limited growth or in the worst cases, in strong deleterious effects to the host organism. Inadequacy of production level has a direct impact on the development of applications when profit margins are narrow, or when treatment and/or disposal of residual matter causes bio-safety or environmental problems. Improvement of the accumulation level of the desired recombinant product thus appears to be one critical factor that warrants commercialization of many applications of molecular farming.

The use of inducible promoters has been proposed, and in some instances used successfully, to counteract the combined effect of all the above-mentioned factors. Strong inducible promoters may succeed in generating high ephemerous transcription rates which result in high transitory accumulation of foreign mRNA and translational product. As a result, when inducibility of expression is paired with adequate synchronized protein recovery procedures, the yield per unit obtained is higher than with the use of constitutive expression.

Several expression cassettes harboring inducible promoters have been developed for microbial production systems, and some are currently available for research purposes. Some inducible promoters are currently used in plant (wound inducibility) or animal (specificity to cells of the mammary glands, PPL) systems, although none reported are using low-cost and bio-safe chemical inducers such as nitrate salts.

It would be highly desirable to be provided with a method of regulating the transcription of transgene in genetically-modified organisms.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a method of regulating the transcription of transgene in genetically-modified organisms.

Another aim of the present invention is to provide the use of expression vectors harboring the coding sequence of a gene of interest under the transcriptional control of promoting sequences for which activity is regulated by the presence of nitrogen.

The present invention relates to the use of a nitrogen-inducible expression cassettes for the controlled expression of foreign genes in plants. It will be shown from the following description that isolating such a regulatory sequences can be performed so that when cis-acting sequences are appropriately associated to the open reading frame of a gene of interest, its transcription can be controlled by the addition of specific nitrogen sources.

In one aspect of this invention, the targeted system uses leguminous plant species, so that constructs containing a nitrate-inducible promoter will be maintained transcriptionally low throughout the growth period if the transgenic plant is maintained on a nitrate-free medium, thus allowing the development of the plant biomass without interference from the transgenic trait. Upon addition of nitrate to the growth medium, transcription will be induced in a relatively large proportion of the biomass over the following days. Optimization of induction time and protein accumulation will then be performed in order to maximize recovery of the desired recombinant product.

Although the following description will make clear that this invention can be easily adapted to nitrate induction on nitrate-deprived transgenic leguminous plants, it should be remembered that this general concept can also be applied to the development of other production systems, making profit of the wide variety of nitrogen assimilation systems in the microbial, plant and animal kingdoms.

In one other aspect of this invention, nitrogen inducibility can also be used to maximize protein production in organisms which do not perform nitrogen fixation through symbiotic association, but that can use variable sources of nitrogen (reduced or oxidized) for growth, and thus possess the ability to develop adequately while one of their nitrogen assimilation pathway is inactive due to lack of one nitrogenous substrate in the growing environment. Using an expression cassettes that controls the transcription of any gene in this inactive pathway in order to drive the

expression of a gene of interest in such an organism, will allow for inducible expression of the transgenic trait upon addition of the previously lacking nitrogenous compound. As an example, duckweed is a plant species that can grow alternately on nitrate or ammonium; this invention could be used to develop an expression cassette harboring an ammonium-inducible promoter appropriately linked to a gene of interest so that the induction would be performed on nitrate-grown transgenic duckweed plants.

In accordance with the present invention there is provided a method for regulating transcription of a foreign gene in transgenic organisms, comprising the steps of:

a) preparing a transgenic organism using an expression construct consisting of at least a nitrogen-inducible promoter having a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof, and an ORF of a gene, wherein said promoter is operationally located with respect to said gene for expression of said gene.

In accordance with a preferred embodiment of the present invention, the method of may further comprise the step of regulating transcriptional expression of said gene by addition or removal of a nitrogen inducer.

In accordance with a preferred embodiment of the present invention, there is provided a method for regulating transcription of a foreign gene in transgenic organisms comprising:

a) preparing an expression construct consisting of at least a nitrogen-inducible promoter with or without cis-acting sequence, an ORF of a gene, and a polyadenylation signal end site at the 3'end of said construct, wherein said promoter is operationally located with respect to said gene for expression of said gene and modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer;
b) sub-cloning the construct of step a) into a suitable transfection vector for said organism;
c) transferring said vector into DNA of said organism or a cell thereof; and

d) selecting for transgenicity on a suitable medium.

In accordance with a preferred embodiment of the present invention, the method of may further comprise the steps of:

e) introducing the vector into a suitable *Agrobacterium tumefaciens* strain;

f) using the *Agrobacterium* strain of step a) to transfer T-DNA into a plant cell;

g) selecting for transgenicity of said plant cell on a suitable medium;

h) regenerating embryos or plantlets from said transgenic cells; and

i) growing mature plants from said regenerated embryos.

In accordance with a preferred embodiment of the present invention, the cis-acting sequence may be isolated from 5' upstream region of an expressed Nir gene in alfalfa.

In accordance with a preferred embodiment of the method of the present invention, the promoter has the sequence set forth in SEQ ID NO:1 to 13 and functional fragments and derivatives thereof.

In accordance with a preferred embodiment of the present invention, the organism is a plant, more preferably a dicotyledonous plant.

In accordance with a preferred embodiment of the present invention, the organism is alfalfa or tobacco.

In accordance with a preferred embodiment of the present invention, the nitrogen inducer is nitrate.

In accordance with a preferred embodiment of the present invention, the DNA transfer method is any suitable transfer method including DNA bombardment, electroporation, PEG-mediated DNA transfer and whiskers, among others.

In accordance with a preferred embodiment of the present invention, the expression construct comprises at least a nitrogen-inducible promoter and at least one cis- or trans-acting elements.

In accordance with a preferred embodiment of the present invention, the organism is a plant, a fungus, a bacteria, a yeast or an animal.

In accordance with a preferred embodiment of the present invention, the promoter or cis-acting sequence is isolated from the 5' upstream region of any gene involved in a nitrogen assimilatory pathway.

In accordance with a preferred embodiment of the present invention, the promoter or cis-acting sequence is isolated from the 5'upstream region of any gene for which transcription is modulated by availability of a given nitrogen source.

In accordance with a preferred embodiment of the present invention, the promoting or cis-acting sequence is any sequence for which transcriptional activity is regulated by addition or removal of any nitrogen source in or from any living organism's environment.

In accordance with a preferred embodiment of the present invention, the organism from which the promoter or cis-acting sequence is isolated from is any plant, fungus, yeast, bacteria or animal.

In accordance with a preferred embodiment of the present invention, there is provided a promoter for promoting transcription of a foreign gene in transgenic organisms, which comprises a nitrogen-inducible promoter with or without cis-acting sequence for expression of said gene and adapted to be modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer.

Preferably, the promoter has a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof.

In accordance with a preferred embodiment of the present invention, there is provided a terminator allowing expression of a foreign gene in transgenic organisms being used in combination with a promoter, which comprises a polyadenylation signal end site for insertion at the 3'end of said gene, wherein said terminator is operationally located with respect to said gene and said promoter and thereby allows expression of said gene.

Preferably, the terminator has a sequence selected from the group consisting of SEQ ID NOS: 14 to 16 and functional fragments and derivatives thereof.

For the purpose of the present invention the following terms are defined below.

The expression "functional fragments or derivatives thereof" is intended to mean any derivative or fragment of sequences SEQ. ID. NOS:1-16 which allow for an equivalent level of expression of a foreign gene as the promoter of the present invention set forth in SEQ. ID. NOS:1-13 or as the terminator of the present invention set forth in SEQ. ID. NOS:14-16.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates GUS expression level using promoter Nir (SEQ ID NOS:2-13) and terminater NOS in the leaves of transgenic tobacco plants before (empty columns) and after (filled columns) nitrate fertilization of the plants. Tobacco plants were transformed with full-length and deletions of the alfalfa NiR promoter and NOS terminator functionally positioned to control transcription and terminaison of the GUS reporter gene, as described in Methods. GUS activity was measured according to Jefferson et al. (1987, *EMBO J.* 13:3901-3907) prior and after induction by nitrate.

Fig. 2 illustrates GUS expression level using promoter Nir (SEQ ID NOS:2, 3, 5-6) and terminater Nir (SEQ ID NOS:15-16) in the leaves of transgenic tobacco plants before (empty columns) and after (filled columns) nitrate fertilization of the plants. Tobacco plants were transformed with full-length and deletions of the alfalfa NiR promoter and NiR terminator functionally positioned to control transcription and terminaison of the GUS reporter gene, as described in Materials and Methods below. GUS activity was measured according to Jefferson et al (1987, *EMBO J.* 13:3901-3907) prior and after induction by nitrate.

Fig. 3 illustrates GUS expression level using promoter NiR and terminator NOS in the leaves of nodulated transgenic alfalfa plants before (empty columns) and after (filled columns) nitrate fertilization. Alfalfa genotype 11.9 was transformed with constructs GC2-E, GC2-B, DC1-D and 35S functionally positioned to drive expression of reporter gene GUS, as described in Khoudi et al (1997, *Gene* 197:343-351). Following regeneration, transgenic plants were transfered to sterile vermiculite and inoculated with Rhizobium strain Balzac (Nitragin). Plants were allowed to grow for 3 weekswith repeated additions of nitrate-free Hoagland's

solution; GUS activity was then measured in first fully expanded leaves as described by Jefferson et al. (1987, *EMBO J.* 13:3901-3907). Plants were then fertilized with 40 mM nitrate for two days. GUS activity was again measured in first fully-expanded leaves. Data shown herein are ratios between post- and pre-nitrate induction GUS specific activities.

DETAILED DESCRIPTION OF THE INVENTION

Following is a detailed description of the method used to generate transgenic tobacco and alfalfa lines that can be modulated in their expression of a reporter gene. It should be remembered that variations could be brought to the method by which nitrogen-inducible promoters could be isolated, by which they could be linked to ORFs in the construct used for expression in plants, by which different cis- and trans-acting elements of the constructs are used and spatially arranged, by which the inducibility by nitrogen is demonstrated and used, while remaining within the scope of this invention.

In this embodiment, a NiR cDNA strand was first isolated from alfalfa using RT-PCR with primers deduced from a consensus plant NiR sequence. This cDNA stretch was then used either to perform upstream/downstream genome walking. The NiR promoter region and deletions, the 5'UTR and the NiR terminator were then positioned functionnally to control transcription and terminaison of reporter gene GUS. These constructs were inserted into suitable expression vectors for DNA bombardment onto tobacco and alfalfa leaves and for *Agrobacterium* mediated DNA transfer as described by Desgagnés et al. (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). These two DNA transfer methods were used to demonstrate that expression of the reporter gene can be modulated by addition or removal of nitrate in the growing medium.

Materials and Methods

Biological material

E. coli strain DH5-α was used to perform all cloning steps. Cold resistant alfalfa genotype 11.9 was used for all experiments including

stable transformation using *A. tumefaciens* infection (Desgagnés et al. (1995, *Plant Cell Tissue Organ Cult.* 42:129-140).

Isolation of total RNA

Total RNA was extracted using a hot phenol method essentially as described by de Vries et al. (1988, B6 page 1, In: Gevin SB and Shilperoot RA editors, *Plant Molecular Biology Manual*, Dordrecht: Kluwer Academic Publisher).

RT-PCR

RT-PCR was used to produce a DNA fragment corresponding to one abundant NiR mRNA molecular species from leaf total mRNA. A conserved region was first identified from 5 public plant NiR ORFs, namely Genbank sequences #AB006032 (Arabidopsis Nir mRNA), # X66145 (Tobacco partial Nir mRNA), #U10419 (Bean complete Nir cds), #X07568 (Spinach Nir mRNA), and #U90429 (Glycine max Nir complete cds). Degenerated oligonucleotides were deduced from two conserved regions, namely Nir5 - 5' GATATTGATGTTAGACTCAAGTGGC 3' (SEQ ID NO:17), at the 5' end and Nir3 - 5' CACYSATTCCACTTCCTWGGC 3' (SEQ ID NO:18), at the 3' end of the coding strand. A reverse transcription reaction was first performed with 200 units of M-MLV reverse transcriptase (RT) for 1 hour at 37°C using 1 μg of total leaf RNA, 4 mM dNTP (1 mM each), 5 μM random hexamer primers in a 1X M-MLV-RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3mM $MgCl_2$). The PCR reaction was performed in a Perkin Elmer Cetus GenAmp PCR system 9600 (EG&G, Wellesley, MA), using 2.5 units of Taq DNA polymerase, 2 μM Nir5 primer, 2 μM Nir3 primer, 800 μM dNTPs (200 μM each) in a 1X PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$). The cycling program used was: an initial 4 min at 94°C, 30 cycles of 1 min at 94°C, 30 sec at 55°C, and 3 min at 72°C. An extension period of 7 min at 72°C was included in the program.

DNA sequencing

DNA sequencing was performed as described by Sanger et al. (1977, *P.N.A.S. USA*, 74:5643-5647).

Genome walking

Walking upstream of the Alfalfa Nir cDNA fragment cloned from the RT-PCR reaction was performed using the Universal Genome Walker Kit from Clontech Laboratories (Palo Alto, CA) (Cat. #1807-1). The NiR-specific custom primers used to amplify sequences upstream of the coding sequence were:

Nir1106r - 5' TTGTCACATCAGCACATCCGTCTTTGC 3' (SEQ ID NO:19), and

Nir1061r - 5' TCGCCAAGTATCTTGTTTGAGCACTTG 3' (SEQ ID NO:20).

The amplified 3775 bp fragment was subcloned into pGEM-T Easy vector (Promega, Madison, WI) (Cat #A1360) for further analysis. The resulting plasmid was named pGNir4c.

The downstream walking was performed as the upstream walking using the following NiR specific primers:

Nir1c - 5' ATGTCTTCCTTCTCAGTACGTTTCCTC 3' (SEQ ID NO:28), and

Nir138c - 5' CAAGTTGATGCATCAAGGTGGGAGCCTAGA 3' (SEQ ID NO:29).

The amplified 3508 bp fragment was subcloned into pGEM-T easy vector (Promega, Madison, WI) (Cat #A1360) for further analysis. The resulting plasmid was named pGN3'1.

Construction of expression cassettes and vectors

The cassettes for expression analysis using the GUS reporter gene were assembled as follows. A promoterless GUS cassette was digested from pBI101 with HindIII and EcoRI, and was inserted into the HindIII and EcoRI sites of the pUC19 polycloning site. The resulting plasmid was named pBI201 and was used for further constructs. The Nir upstream sequences were PCR amplified using the AP2 primer from the Universal Genome Walking Kit as upstream positioned primer, and either one of custom-designed downstream primers ending with a SmaI

restriction site. The 4 primers were positioned either in the 5' UTR region of the gene (Nir-23r-Sma-5'AGAGCCCGGGAGAAGAGAGTGTGTTTG3' (SEQ ID NO:21)), at the end of the transit peptide coding sequence (Nir51r-Sma - 5' TTCTCCCGGGGGACGAGAGATGGATGGT 3' (SEQ ID NO:22)), 50 bp after the transit peptide coding sequence (Nir103r-Sma - 5' TTCTCCCGGGGTTGAA-ACAGGTGCAACTGA 3' (SEQ ID NO:23)), and 100 pb after the transit peptide coding sequence (Nir158r-Sma - 5' TTCTCCCGGGTAACCATCTTTTTCCTCA 3' (SEQ ID NO:24) Amplification was performed under standard conditions with pGNir4c plasmid as template.

The amplified fragments were digested with specific restriction enzymes in order to produce 5' deletions of the Nir promoter. The pNir3k-23 was produced by digesting the fragment previously amplified by AP2 and Nir-23r-Sma primers with XmaI, and inserting the resulting fragment into pBI201 previously digested with XmaI. A similar strategy was used to produce the pNir3k51, pNir3k103, and pNir3k158 plasmids except that the downstream primers used were Nir51r-Sma, Nir103r-Sma, and Nir158r-Sma, respectively. The pNir2.2k-23 was produced from a SmaI-BglII digestion of the AP2 - Nir-23-Sma amplified fragment inserted into the pBI201 previously digested with SmaI and BamH1. The same strategy was used to produce the pNir2.2k51, pNir2.2k103, and pNir2.2k158 plasmids except that the downstream primers used were Nir51r-Sma, Nir103r-Sma, and Nir158r-Sma, respectively. Fidelity and orientation of the insertions were verified by digestion with restriction enzymes. These deletion fragments were ligated to the 5'terminus of the GUS reporter gene in pBI201, and used for transitory expression studies using DNA bombardment. Upon identification of the adequate deletion fragments, they were sub-cloned into a binary plant expression vector such as pBI101 (Clonetech).

For the construction of the cassettes containing the NiR terminator downstream of the GUS gene in addition to the NiR promoter, the following NiR specific primers were used:

Nir2514c-Sac - 5' AGAAGAGCTCAGTATATAGGTATTTGGTGA 3'(SEQ ID NO:30)

Nir2728c-Sac - 5' AGAAGAGCTCTTGTACATTTGGATAAGTCA 3' (SEQ ID NO:31)
Nir3029r-Eco - 5' AGAAGAATTCGTTTTCCCGATACTTCAACT 3' (SEQ ID NO:32)

A 617 bp terminator fragment was PCR amplified using the primers Nir2514c-Sac and Nir3029r-Eco, and a 503 bp terminator fragment was PCR amplified using the primers Nir2728c-Sac and Nir3029r-Eco. The fragments obtained were digested with SacI and EcoRI and inserted into the plasmids containing the NiR-GUS constructs after deletion of the NOS terminator between the SacI and EcoRI sites.

These recombinant plasmids were used for stable integration through *A. tumefaciens* infection as described below.

***Agrobacterium*-mediated DNA transfer and regeneration of transgenic alfalfa lines**

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described in Khoudi et al (1997, *Gene* 197:343-351). Selected *Agrobacterium* strains were then co-cultivated with leaf disks from genotype C5-1 for 4 days in the absence of selection pressure (kanamycin). Following this incubation period, leaf disks were washed and pampered, and then allowed to form calli onto medium B5H. Calli were then transferred for 21 days on SH medium for embryo induction and for 28 days on BOi2Y for embryo development. Torpedo-shaped embryos were removed from Boi2Y and placed on MS medium for regeneration. Kanamycin was present in all cultivation medium except for co-cultivation and regeneration on MS. This method is described in length in Desgagnés et al (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). Rooted plantlets were grown to maturity in the greenhouse. Integration of the transgene was verified by PCR amplification of a NiR-GUS fragment from genomic DNA. The primers used were:

Nir-102c – 5' CACACTTCTTCACTCACCTCTCAA 3' (SEQ ID NO:25)
Nir-2016c – 5' ATCTAGGAGGGGCAGACATTG 3' (SEQ ID NO:26)
GUS228r – 5' TCGGTATAAAGACTTCGCGCTGAT 3' (SEQ ID NO:27)

Agrobacterium-mediated DNA transfer and regeneration of transgenic tobacco lines

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described in Khoudi et al (1997, *Gene* 197:343-351). Selected strains were co-cultivated with leaf disks for 2 days on MS medium without kanamycin. After this period, the explants were transferred to the selection medium (MS with Kanamycin). The explants were kept on this medium for 3 weeks to allow the formation of calli and shoots from the transfected cells. The kanamycin resistant shoots were transferred into the rooting MS medium. Rooted plantlets were grown to maturity in the greenhouse. Integration of the transgene was verified by PCR amplification of a NiR-GUS fragment from genomic DNA. The primer used were:

Nir-102c – 5' CACACTTCTTCACTCACCTCTCAA 3' (SEQ ID NO:25)
Nir-2016c – 5' ATCTAGGAGGGGCAGACATTG 3' (SEQ ID NO:26)
GUS228r – 5' TCGGTATAAAGACTTCGCGCTGAT 3' (SEQ ID NO:27)

Nitrate induction

Transgenic and non-transgenic tobacco and alfalfa plants were grown in vermiculite medium without nitrate. Mineral balance was kept by repeated additions of nitrate-free Hoagland's solutions (Hoagland and Varnon, 1950, Circular 347, *California Agr. Exp. Stat. Berkeley*). Nitrate induction was performed by watering the plants with 20-20-20 fertilizer at a concentration of $5gL^{-1}$ or as an alternative with Hoagland's solution supplemented with 40 mM nitrate.

NiR promoter activity in tobacco leaves

The NiR derived promoters were placed upstream of the GUS reporter gene in transcriptional and translational fusions. The 5' deletions of the NiR promoter analyzed here consisted in (1) a putative full length promoter comprising 2813 bp upstream of the initial ATG of the coding region, (2) a 1905 bp version of the promoter, and (3) a shorter 1111 bp version of the promoter. The 3' end of the promoter was fused to the 5' end of the GUS coding region to form transcriptional and translational

fusions. Translational fusions analyzed allowed the production of ß-glucuronidase containing (1) the NiR chloroplast transit peptide, (2) the NiR transit peptide with an additional 17 amino acids from NiR, and (3) the NiR transit peptide with an additional 36 amino acids from NiR. The twelve combinations of 5' and 3' deletions of the NiR promoter introduced into tobacco plants are presented in Fig. 1.

The gene constructs were transferred into tobacco plants using the *Agrobacterium*-mediated transfection method (Khoudi et al., 1997, *Gene* 197:343-351). Transgenic plants were transferred to growth chambers and analyzed for their leaf ß-glucuronidase content before and after nitrate fertilization. Fig. 1 presents the median level of ß-glucuronidase activity measured in the 1st expanded leaf of plantlets.

All the NiR derived promoters showed reactivity to nitrate induction. Between 5 and 10 fold increase of ß-glucuronidase expression was generally observed, irrespectively of the promoter truncation, indicating that important nitrate responsive elements are contained within the first 1.1 kb upstream of the initial ATG. Both 5' and 3' deletions of the NiR promoter led to important modifications of ß-glucuronidase activity. The highest level of GUS expression was obtained with the 2.8 kb promoter, indicating that the far upstream regions have a regulatory role for the level of NiR expression in the leaves.

The translational fusions of the promoter to the GUS coding region resulted in variable expression level depending on the extension of the 5' end of the promoter. However, the shortest fusion (containing the 17 a.a. NiR transit peptide fused to the amino-terminal end of the ß-glucuronidase) constantly resulted in the highest level of activity for all three 5' end truncations. This short translational fusion, combined with the longest extension of upstream promoter regions gave rise to the strongest promoter (3kb+50). When induced, this specific construct resulted in more than 13-fold the level of GUS expression obtained with the 35S-GUS-NOS construct.

When considering the longest 5' extension of the promoter, the transcriptional fusion to the GUS gene (3kb-5) was ~7 times less effective than the short translational fusion (3kb+50). However, in it's induced state, the level of GUS expression in the plants harboring the 3kb-5 promoter deletion was more than 1,8-fold that observed in the 35S-GUS-NOS plants.

Taken together, the results presented here clearly indicate that the sequences upstream of the alfalfa NiR gene have the capacity to drive high and inducible expression of an exogenous gene in tobacco leaves.

Efficiency of NiR terminator

Tobacco plants were transformed with constructs consisting of promoter NiR and deletions (SEQ ID NOS: 2, 3, 5 and 6), and 35S, together with 3'UTR sequences and terminator (SEQ ID NOS: 15 and 16), functionally positioned to drive transcription and termination of reporter gene GUS. Growth, nitrate induction and GUS activity measurements were performed as per experiment illustrated in Fig 1. Results shown in Fig 2 demonstrate that the terminator sequence of Nir allows termination of transcription into a translatable messenger RNA.

NiR promoter activity in alfalfa leaves

Transgenic alfalfa plant containing the gene constructs presented in Fig. 3 were obtained using the *Agrobacterium*-mediated transfection method of Desgagnés et al. (1995, *Plant Cell Tissue Organ Cult.* 42:129-140). The *in vitro* plants were transferred into growth chamber to allow a normal vegetative growth. Cuttings from each transgenic line were grown in vermiculite and fertilized with nitrate-free Hoagland medium. After two weeks, the roots were inoculated with Nitragin (LiphaTech inc., Milwaukee, WI). Two weeks after inoculation, nodules had developed on the roots. Nodulated plants were allowed to continue their vegetative growth for at least another week before the fluorometric measurement of ß-glucuronidase activity (before induction)

was performed. After the measurement, the plants were watered with Hoagland medium containing 40 mmol KNO_3. Two days after induction, leaf ß-glucuronidase activity was measured to evaluate the nitrate inducibility of the NiR promoter in alfalfa leaves. Results are presented in Fig. 3. Results show that promoter NiR induces expression of GUS reporter gene upon addition of nitrate in nodulated alfalfa plants. Taken together, this last series of result demonstrate that NiR promoter inducibility can be used to positively regulate expression of a foreign gene in alfalfa plants when fixation of atmospheric nitrogen is replaced by nitrate assimilation.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for regulating transcription of a foreign gene in transgenic organisms, comprising the steps of:
   a) preparing a transgenic organism using an expression construct consisting of at least a nitrogen-inducible promoter having a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof, and an ORF of a gene, wherein said promoter is operationally located with respect to said gene for expression of said gene.

2. The method of claim 1, which further comprises the step of regulating transcriptional expression of said gene by addition or removal of a nitrogen inducer.

3. A method for regulating transcription of a foreign gene in transgenic organisms comprising:
   a) preparing an expression construct consisting of at least a nitrogen-inducible promoter with or without cis-acting sequence, an ORF of a gene, and a polyadenylation signal end site at the 3'end of said construct, wherein said promoter is operationally located with respect to said gene for expression of said gene and modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer;
   b) sub-cloning the construct of step a) into a suitable transfection vector for said organism;
   c) transferring said vector into DNA of said organism or a cell thereof; and
   d) selecting for transgenicity on a suitable medium.

4. The method of claim 3, wherein said organism is a plant.

5. The method of claim 4, which further comprises the steps of:
   e) introducing the vector into a suitable *Agrobacterium tumefaciens* strain;

f) using the *Agrobacterium* strain of step a) to transfer T-DNA into a plant cell;

g) selecting for transgenicity of said plant cell on a suitable medium;

h) regenerating embryos or plantlets from said transgenic cells; and

i) growing mature plants from said regenerated embryos.

6. The method of claim 3, wherein the cis-acting sequence is isolated from 5' upstream region of an expressed Nir gene in alfalfa.

7. The method of claim 3, wherein the promoter has the sequence set forth in SEQ ID NOS:1 to 13 and functional fragments and derivatives thereof.

8. The method of claim 1, wherein the organism is selected from the group consisting of a plant, a fungus, a bacteria, a yeast and an animal.

9. The method of claim 8, wherein the plant is a dicot.

10. The method of claim 9, wherein the dicot is alfalfa or tobacco.

11. The method of claim 2, wherein the nitrogen inducer is nitrate.

12. The method of claim 3, wherein the DNA transfer method is any suitable transfer method including DNA bombardment, electroporation, PEG-mediated DNA transfer and whiskers.

13. The method of claim 3, wherein the expression construct comprises at least a nitrogen-inducible promoter and at least one cis- or trans-acting elements.

14. The method of claim 3, wherein the promoter or cis-acting sequence is isolated from the 5' upstream region of any gene involved in a nitrogen assimilatory pathway.

15. The method of claim 3, wherein the promoter or cis-acting sequence is isolated from the 5'upstream region of any gene for which transcription is modulated by availability of a nitrogen source.

16. The method of claim 3, wherein the promoting or cis-acting sequence is any sequence for which transcriptional activity is modulated by addition or removal of any nitrogen source in or from any living organism's environment.

17. The method of claim 3, wherein the organism from which the promoter or cis-acting sequence is isolated from is any plant, fungus, yeast, bacteria or animal.

18. A promoter for promoting transcription of a foreign gene in transgenic organisms, which comprises a nitrogen-inducible promoter with or without cis-acting sequence for expression of said gene and adapted to be modulated for transcriptional expression of said gene by addition or removal of a nitrogen inducer.

19. The promoter of claim 18, wherein said promoter has a sequence selected from the group consisting of SEQ ID NOS: 1 to 13 and functional fragments and derivatives thereof.

20. A terminator allowing expression of a foreign gene in transgenic organisms being used in combination with a promoter, which comprises a polyadenylation signal end site for insertion at the 3'end of said gene, wherein said terminator is operationally located with respect to said gene and said promoter and thereby allows expression of said gene.

21. The terminator of claim 19, wherein said terminator has a sequence selected from the group consisting of SEQ ID NOS: 14 to 16 and functional fragments and derivatives thereof.

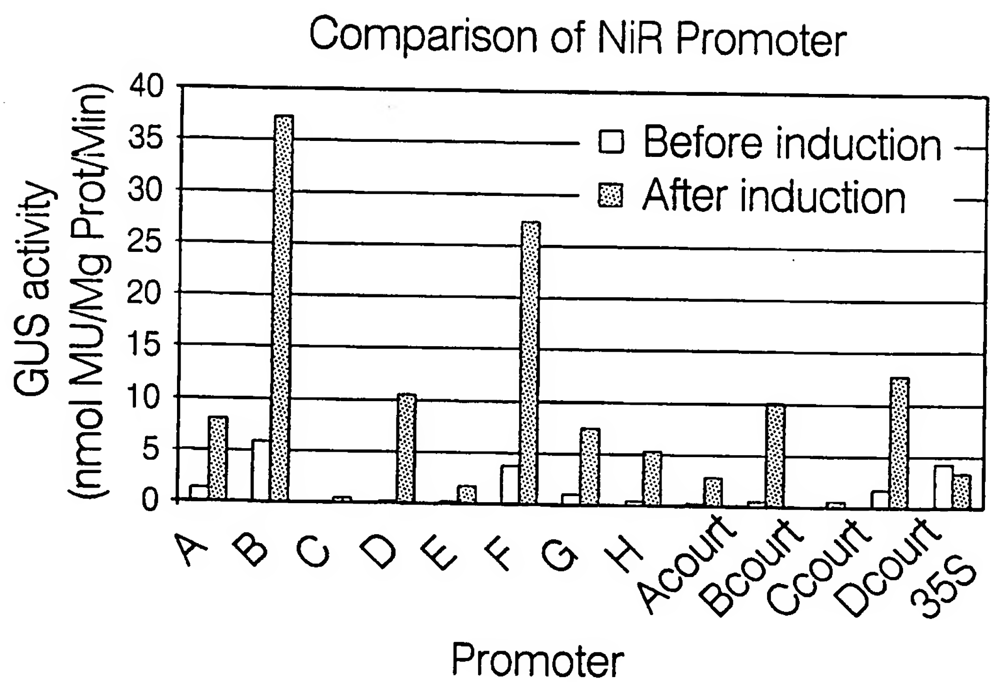
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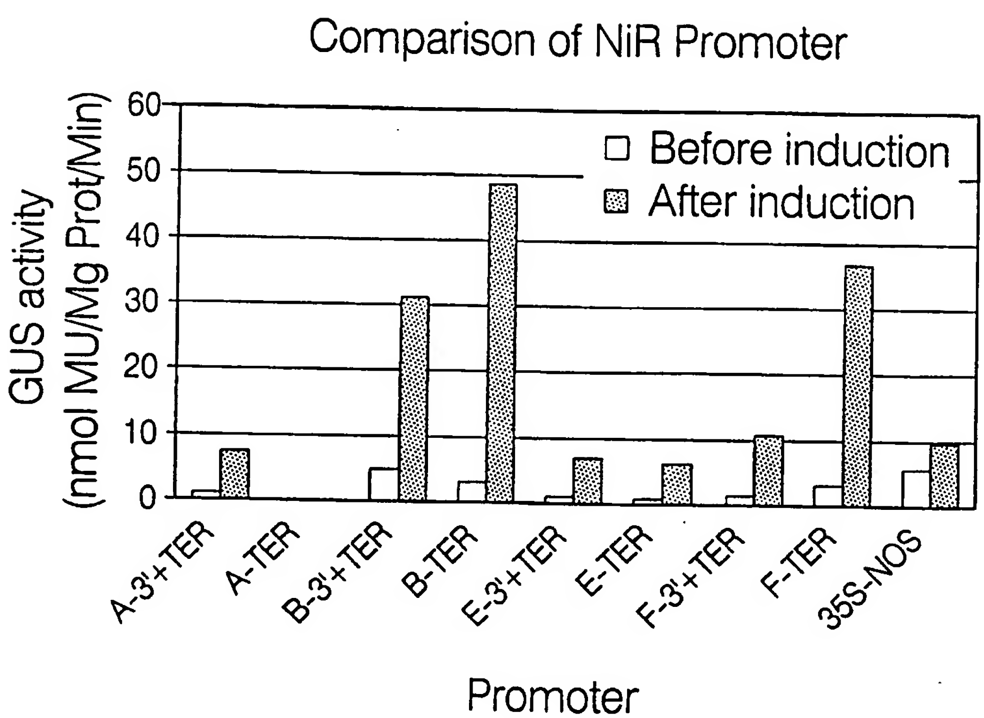
Fig. 1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After induction
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Fig. 2

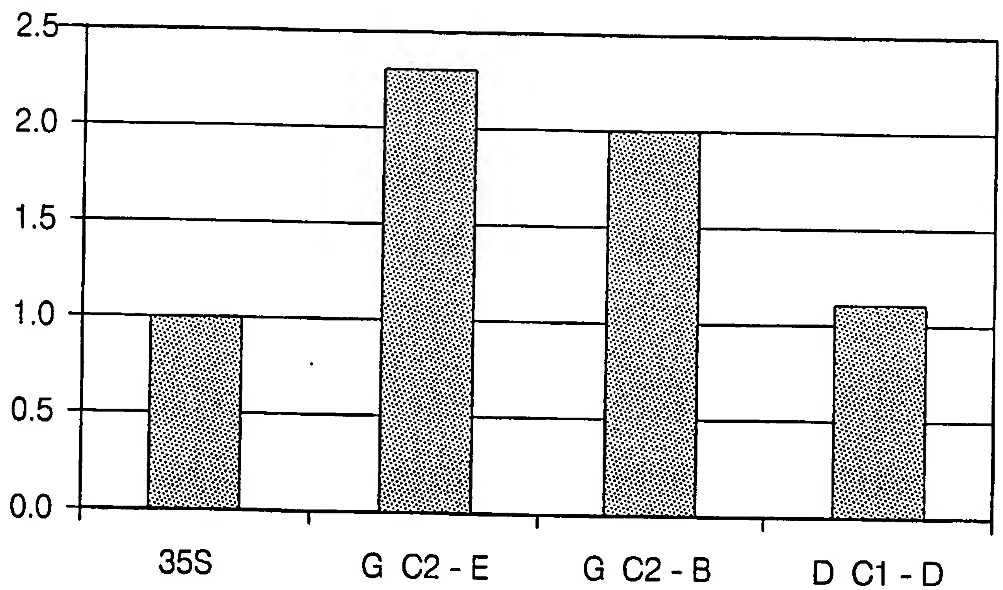

Fig. 3

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MEDICAGO INC.

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<210> 5
<211> 2863
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating expression of foreign genes

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atcatatcta tgtgagagaa aggggaacat ttgagatgga gtccatttac ttgaggtata 180

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acttgatggc atctcttggt ttctttggca atcacatgcc taagaaataa atagtatcat     360
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ctctggcaat catatgccta agaaataaat agtatcatat gattgtgttt ggtcagactt     540
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<210> 6
<211> 2124
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 6

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atgtcatgag ataacataat gataacacat tattttgaca caaatgggca gatctagcaa     180
tttaactctg gagtccttca agactgctgt tcttacgaag ttcacgtccc tgaatcatgt     240
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tcctgtatgg aagcctgaaa gacctcaaat tctaaaaggt ggcgataaat tgaaggttta     300
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ggatgttgat ttcagaagtc acttggagag caatccttgt gccaagtttg aagtaatttt     420
tgtgtagcat atgttgagct acctacaatt tacatgatca cctagcatta gctctttcac     480
ttaactgaga gaatgaagtt ttaggaatga gtatgaccat ggagtcggca tggctttgta     540
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caaccatact taaacccctt tttgtaagat aactgaatgt tcatatttaa tgttgggttg     660
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<210> 7
<211> 1160
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 7

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agagataaag catactaatt tgactcttgg aaacccataa agtttatgtt atccgtgttc     180
tggaccaatc cacttggggg cataacctgt gtctatgtgt ggtttggttt ccattctgat     240
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<210> 8
<211> 2904
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating expression of foreign genes

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atctgctgca actccatcag ttgc                                           2904

      &lt;210&gt; 9
      &lt;211&gt; 2165
      &lt;212&gt; DNA
      &lt;213&gt; Artificial Sequence

      &lt;220&gt;
      &lt;223&gt; Sequences to be used as promoter for regulating
            expression of foreign genes

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atgtcatgag ataacataat gataacacat tattttgaca caaatgggca gatctagcaa     180
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gttgc                                                                2165

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      &lt;211&gt; 1316
      &lt;212&gt; DNA
      &lt;213&gt; Artificial Sequence

      &lt;220&gt;
      &lt;223&gt; Sequences to be used as promoter for regulating
            expression of foreign genes
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caatccactt gggggcataa cctgtgtcta tgtgtggttt ggtttccatt ctgatttatg 360
cggcgacttg taatttaaaa tctaggaggg gcagacattg aacaatccca atattttaat 420
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<211> 2971
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating
expression of foreign genes

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<210> 12
<211> 2232
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 12

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atgtcatgag ataacataat gataacacat tattttgaca caaatgggca gatctagcaa    180
tttaactctg gagtccttca agactgctgt tcttacgaag ttcacgtccc tgaatcatgt    240
tcctgtatgg aagcctgaaa gacctcaaat tctaaaaggt ggcgataaat tgaaggttta    300
caaaatatac cctgcgggct tgacacagag gcaagctctt tataccttcc agttcaacgg    360
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aaagatggtt ac 2232

<210> 13
<211> 1383
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as promoter for regulating expression of foreign genes

<400> 13
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caactcgttc cggggccgct acaactgaaa caaaagaaga tattttctct ctcttcagaa 180
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taaagcatac taatttgact cttggaaacc cataaagttt atgttatccg tgttctggac 300
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cggcgacttg taatttaaaa tctaggaggg gcagacattg aacaatccca atattttaat 420
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<210> 14
<211> 3472
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as terminator for regulating expression of foreign genes

<400> 14

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gaaagctcta agcttactaa agatgatatt gatgttagac ttaaatggct tggtcttttt 240
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<210> 15

<211> 617
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as terminator for regulating
expression of foreign genes

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tgaagtatcg ggaaaac 617

<210> 16
<211> 502
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as terminator for regulating
expression of foreign genes

<400> 16
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aataaatttt attttatttc aaggaccaga ttttataagg aaaccgctaa tctaactatc 120
tttactcgta atttgtcatt tgagagctac ggagatcgtt gagtttacgt atgagtgttt 180
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<210> 17
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 17
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<210> 18
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 18
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<210> 19
<211> 27
<212> DNA
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<220>
<223> Sequences to be used as primers

<400> 19
ttgtcacatc agcacatccg tctttgc 27

<210> 20
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 20
tcgccaagta tcttgtttga gcacttg 27

<210> 21
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 21
agagcccggg agaagagagt gtgtttg 27

<210> 22
<211> 28
<212> DNA
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<220>
<223> Sequences to be used as primers

<400> 22
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<210> 23
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 23
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<210> 24
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<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 24
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<210> 25
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 25
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<210> 26
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Sequences to be used as primers

<400> 26
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<210> 27
<211> 24
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<220>
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<400> 27

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<210> 28
<211> 27
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<220>
<223> sequences to be used as primers

<400> 28
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<210> 29
<211> 30
<212> DNA
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<220>
<223> sequences to be used as primers

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<210> 30
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<210> 31
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